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Although prostate cancer (PC) is the most common cancer in the western countries, risk factors contributing to the development and progression of this disease have not been well characterized. Furthermore, research on genetic susceptibility to prostate cancer is in its infancy. This study has built upon ongoing NCI-funded projects by adding a panel of genetic susceptibility markers. Specifically, as a part of this study, we were able to accrue an additional 140 new patients with metastatic disease. We evaluated constitutional markers of genetic susceptibility as predictors of prostate cancer risk including: a) polymorphisms within the androgen receptor, 5-α-reductase and vitamin D receptor genes, b) relative expression levels of several mismatch repair genes (*hMSH2* and *hMLH1*) and radiosensitivity related genes (*ATM, GADD45, XRCC1*), and c) frequency of replication errors in tumor and normal DNA. These genetic susceptibility marker data are being integrated with epidemiologic and clinical information. Results from this research may identify markers of progression, both epidemiologic and molecular, which could help in the diagnosis and treatment of prostate cancer. Our findings suggest decreased mismatch repair gene expression may be associated with increased risk of prostate cancer. These results suggest that DNA damage-repair pathways may be involved in prostate carcinogenesis. Results from the analyses of the genetic polymorphisms suggest that some of these may also play a role in prostate cancer prognosis. Further and larger studies are needed to confirm these findings and to further explore the molecular basis of the underlying mechanisms of prostate cancer etiology and progression.

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DAMD17-98-1-84711 (Sara S. Strom, Ph.D.)

A Molecular Epidemiologic Case-Case Study of Prostate Cancer Susceptibility

INTRODUCTION

Although prostate cancer (PC) is the most common cancer in the western countries, risk factors contributing to the development and progression of this disease have not been well characterized. Furthermore, research on genetic susceptibility to prostate cancer is in its infancy. This study has built upon ongoing NCI-funded projects by adding a panel of genetic susceptibility markers. Specifically, as a part of this study, we were able to accrue an additional 140 new patients with metastatic disease. We evaluated constitutional markers of genetic susceptibility as predictors of prostate cancer risk including: a) polymorphisms within the androgen receptor, 5-α-reductase and vitamin D receptor genes, b) relative expression levels of several mismatch repair genes (*hMSH2* and *hMLH1*) and radiosensitivity related genes (*ATM, GADD45, XRCC1*), and c) frequency of replication errors in tumor and normal DNA. These genetic susceptibility marker data are being integrated with epidemiologic and clinical information. Results from this research may identify markers of progression, both epidemiologic and molecular, which could help in the diagnosis and treatment of prostate cancer. ***By adding patients with metastatic disease, a new set of markers, and by using an ongoing study protocol, data collection instruments, and data management tools currently in operation, we have incorporated patients with the entire spectrum of disease in a time- and cost-effective manner.***

STATEMENT OF WORK

Task 1 **Subject Recruitment.** (Months 1-24)

A. Identify and enroll 100 prostate cancer patients with metastatic disease from UTMDACC.

Through the end of the funding period, we have identified and enrolled 140 men with metastatic prostate cancer (80% Caucasian, 12% African-American and 8% Hispanic) in this study.

B. Complete interviews, anthropometric measurements, and collect blood samples and tissue from study participants.

Interviews have been completed for all participants, and blood samples have been collected for 98% of participants.

C. Abstract medical records.

Medical records for all accrued patients have been abstracted.

D. Complete data-entry using protocols developed for the on-going study.

Data have been entered using the previously developed protocols for all study participants.

E. Perform quality control checks using protocols developed for the ongoing study.

Data quality control checks have been updated and conducted in accordance with the existing protocols.

Task 2 **Evaluate Constitutional Markers of Genetic Susceptibility.** (Months 1-24)

A. DNA will be extracted from peripheral blood samples (200 from ongoing study and 100 newly-enrolled metastatic cases) by the UTMDACC Institutional Molecular Core.

DNA has been extracted for all study participants as previously proposed.

B. Complete analysis of polymorphisms related to androgen metabolism (P. Thompson)

Analyses of the polymorphic genes associated with androgen metabolism (i.e., 5-alpha reductase, androgen receptor) and Vitamin D receptor have been completed and are reported below.

Vitamin D Receptor Polymorphism (VDR)

We analyzed the VDR TaqI restriction fragment polymorphism in codon 352 of the vitamin D receptor gene for 407 Caucasian cases and 403 age- and ethnicity- frequency-matched controls using a polymerase chain reaction (PCR) based method. These data were preliminarily presented at the 2000 American Association for Cancer Research meeting and have recently been submitted for publication (Appendix A). Depending on the presence or absence of the TaqI restriction site, individuals were classified as having TT, Tt, or tt genotype.

We did not find any significant association between the presence of the TaqI allele and overall PC risk. While we did find that the OR was slightly higher in the younger age group ($\leq$ 55 years) relative to the older group (>55 years) (OR = 1.59, p=0.31 and OR = 1.21, p=0.35, respectively), this difference was not significant. We also found evidence suggestive of an association between the presence of the TaqI allele with metastatic PC compared to locally confined disease (OR = 1.36, p = 0.28 and OR = 1.22, p = 0.34, respectively), however, again these results were not statistically significant.

5-α-reductase Polymorphism (SRD5A2)

We analyzed this polymorphism in association with known clinical prognostic indicators among 280 Caucasian men with all stages of PC enrolled in our ongoing PC studies. DNA was isolated from peripheral lymphocytes and genotyped for the single nucleotide polymorphism in the SRD5A2 gene that results in the substitution of Valine for Leucine at codon 89 (V89L). Overall, 8% of out patients had the Leu/Leu genotype. Men with the Leu/Leu genotype tended to be older than those with Val/Val or Val/Leu genotypes (Table 1) and had lower combined Gleason scores compared to those with Val/Val or Val/Leu (Table 1). We also found that men with metastatic PC (N = 66) were slightly less likely to have the Leu/Leu genotype relative to men who were diagnosed with non-metastatic (N = 214) disease (Table 2). These data were preliminarily presented at the 2001 AACR meeting (Appendix B). We are currently working on a manuscript for submission.

Table 1. Patient Characteristics by SRD5A2 Genotype

Characteristic	Val/Val N = 133	Val/Leu N = 126	Leu/Leu N = 21	P-value
Age @ diagnosis	61.7	61.5	62.5	0.8
Education (years)	15.7	15.2	14.1	0.06
Smoking history				
Current	6 (5%)	11 (9%)	4 (19%)	
Former	87 (65%)	78 (62%)	15 (71%)	
Never	40 (30%)	37 (29%)	2 (10%)	0.07
Family history of PC*				
Yes	25 (19%)	29 (23%)	3 (14%)	
No	108 (81%)	97 (77%)	18 (86%)	0.5
Disease Stage				
Non-metastatic	100 (75%)	97 (77%)	17 (81%)	
Metastatic	33 (25%)	29 (23%)	4 (19%)	0.8
Gleason score (mean)	7.2	7.1	6.8	0.3

* Family history of PC among first degree relatives (e.g., father, brothers and sons)

Table 2. Patient Characteristics by PC Stage

Characteristic	Overall (N = 280)	Non-Metastatic (N = 214)	Metastatic (N = 66)	P- value**
Age @ diagnosis	61.7	62.0	60.7	0.2
Education (years)	15.4	15.4	15.2	0.7
Smoking history				
Current	21 (8%)	13 (6%)	8 (12%)	
Former	79 (28%)	136 (64%)	44 (67%)	
Never	180 (64%)	65 (30%)	14 (21%)	0.1
Family History of PC*				
Yes	57 (20%)	41 (19%)	16 (24%)	
No	223 (80%)	173 (81%)	50 (76%)	0.4
5-α-reductase genotype				
Val/Val	133 (48%)	100 (47%)	33 (50%)	
Val/Leu	126 (45%)	97 (45%)	29 (44%)	
Leu/Leu	21 (7%)	17 (8%)	4 (6%)	0.8
Gleason score (mean)	7.1	6.9	8.0	< 0.001

* Family history of PC among first degree relatives (e.g., father, brothers and sons)
** P-values calculated for Non-metastatic Cases vs. Metastatic Cases

Table 3. Patient Characteristics by PC Stage & SRD5A2 Genotype

	Val/Val		Val/Leu		Leu/Leu	
	Non-Met	Metastatic	Non-Met	Metastatic	Non-Met	Metastatic
	N = 100	N = 33	N = 97	N = 29	N = 17	N = 4
Age @ diagnosis	62.2	60.4	61.7	60.8	62.7	62.0
Education (years)	15.7	15.5	15.3	15.1	14.0	14.5
Smoking history						
Current	4 (4%)	2 (6%)	5 (5%)	6 (21%)	4 (24%)	0 (0%)
Former	65 (65%)	22 (67%)	59 (61%)	19 (66%)	12 (71%)	3 (75%)
Never	31 (31%)	9 (27%)	33 (34%)	4 (14%)	1 (6%)	1 (25%)
Family history of PC*						
Yes	19 (19%)	6 (18%)	19 (20%)	10 (35%)	3 (18%)	0 (0%)
No	81 (81%)	27 (82%)	78 (80%)	19 (65%)	14 (82%)	4 (100%)
Gleason score (mean)	6.9	8.2	6.8	7.9	6.7	7.3

* Family history of PC among first degree relatives (e.g., father, brothers and sons)

As shown in Table 3 above, these data suggest a role in PC progression for the SRD5A2 V89L polymorphism. Men with the Leu/Leu were diagnosed with lower grade prostate cancer at a later age and were less likely to be diagnosed with metastatic disease than men with the Val/Val or Val/Leu genotype (Table 3).

Androgen Receptor Polymorphisms (AR)

We evaluated the number of trinucleotide repeats for two regions contained in exon 1 of the AR gene. These regions have been both previously identified to be associated with risk of developing prostate cancer. Using our expanded sample set, we genotyped DNA isolated from peripheral lymphocytes for 686 Caucasian, 76 Hispanic and 126 African-American men with PC. We found evidence consist with the existing literature that African-American men tend to have shorter repeat segments of both trinucleotides we evaluated, CAG and GGC (Table 4). There was also suggestion of an association between younger age at diagnosis and shorter repeat length for both trinucleotides (data not shown). These data are being prepared for submission as a manuscript.

Table 4. AR Trinucleotide Repeat Length by Ethnicity

	Caucasian (n = 686)	**Hispanic (n = 76)**	**χ^2 P – value***	**African-American (n = 126)**	**χ^2 P – value****
Androgen Receptor (CAG) repeat lengths					
Long (24+)	220 (32.1%)	21 (27.6%)		21 (16.7%)	
Intermediate (21-23)	243 (35.4%)	31 (40.8%)		37 (29.4%)	
Short ($\leq$ 20)	223 (32.5%)	24 (31.6%)	0.6	68 (54.0%)	< 0.001
Mean (CAG) repeat length (min-max)	22 (7-34)	22 (12-29)		20 (11-29)	
Androgen Receptor (GGC) repeat lengths					
Long (18+)	221 (32.2%)	22 (28.9%)		39 (31.2%)	
Intermediate (17)	395 (57.6%)	42 (55.3%)		16 (12.8%)	
Short (<17)	70 (10.2%)	12 (15.8%)	0.3	70 (56.0%)	< 0.001
Mean (GGC) repeat length (min-max)	17 (4-21)	17 (5-19)		16 (8-20)	

*χ^2 p –value for Hispanics vs. Caucasians
** χ^2 p –value for African-Americans vs. Caucasians

C. Determine expression levels of DNA mismatch repair and radiosensitivity related genes (Q. Wei)

Using a novel multiplex RT-PCR assay, we determined mismatch repair gene expression levels among 70 prostate cancer cases and 97 healthy controls. These results have been summarized in a manuscript published in a peer-reviewed journal (see Appendix C). Overall, the cases had lower expression levels for these genes than did healthy controls. The data below summarize these findings.

Table 5. Mismatch Repair Gene Expression: case-control

Gene	Expression Level*	Number		OR (95% CI)
		Cases (n =70)	Controls (n = 97)	
hMLH1	HT	17	32	1.00
	MT	15	33	0.86 (0.37-2.00)
	LT	38	32	2.24 (1.05-4.75)
hMSH2	HT	8	32	1.00
	MT	25	33	3.03 (1.19-7.70)
	LT	37	32	4.62 (1.87-11.46)

**HT, highest tertile; MT, middle tertile; LT lowest tertile; based on controls levels*

We also used similar techniques to examine for possible differences in radiosensitivity genes, GADD45, XRCC1, and ATM. This manuscript is in preparation.

Table 6. Radiosensitivity Gene Expression: case-control

Gene	Expression Level*	Number		OR (95% CI)
		Cases (n =70)	Controls (n = 97)	
GADD45	HT	7	32	1.00
	MT	16	33	2.22 (0.81-6.10)
	LT	47	32	6.71 (2.64-17.07)
XRCC1	HT	19	32	1.00
	MT	19	33	0.97 (0.44-2.16)
	LT	32	32	1.68 (0.80-3.57)
ATM	HT	11	32	1.00
	MT	14	33	1.23 (0.49-3.12)
	LT	45	32	4.10 (1.80-9.30)

**HT, highest tertile; MT, middle tertile; LT lowest tertile; based on controls levels*

Task 3 **Determine Microsatellite Instability in Tissue Samples.** (Months 1-24)

A. DNA will be extracted from tumor and normal tissue in a subset of 120 cases (40 insignificant, 40 significant, and 40 metastatic PC).

B. Determine the frequency of replication errors.

DNA has been extracted from tumor and normal tissue in a subset of cases to determine the frequency of replication errors.

For identifying microsatellite instability (MSI) at a given locus, we used the definition of MSI recommended at "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" held in Bethesda, MD, December 8-9, 1997. MSI was defined as any length change, due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue (Boland *et al.*). It was stressed that MSI, as defined above, does not describe a particular tumor phenotype, but refers only to the observation of instability at a given marker. One of our goals in this proposal was to evaluate these markers in order to determine if they are useful in detecting MSI in PC.

For colorectal cancer, several studies have shown that a subset of colorectal cancers demonstrates the phenomenon of MSI and that such tumors can be divided into three groups according with the frequency of instability (Thibodeau *et al.*, 1998).

The panel of five markers that were recommended by the workshop were two mononucleotide repeats (*BAT25, BAT 26*) and three dinucleotide repeats (*D2S123, D5S346, and D17S250).* A tumor was defined as MSI-H if instability was detected in two or more of these markers. They were MSI-L if only one of the five markers displayed instability, and if none of the markers displayed instability, they were classified as MSI-L-MSS. We tested this panel of markers on 25 PC.

For the collection of tissue for the present study, normal and tumor sample were obtained from different regions of the sections of tissue by means of microdissection identified by Dr. Patricia Troncoso, a pathologist at our institution. DNA was extracted from formalyn fixed tissue using a Qiagen kit for DNA extraction from formalyn fixed tissue. We tested for MSI using the NCI panel. Following PCR, the samples were analyzed on an automated ABI model 373 DNA sequencer equipped with gene scan software.

We decided to test 25 PC to obtain preliminary data. Three tumors displayed MSI at one locus and were therefore classified MSI-L. For two of these, the locus displaying the instability was *BAT25*, for the other tumor, the instability was observed in *DS17S250*. In one of the patients with a tumor displaying instability at *BAT25*, he had a second tumor which displayed instability at two loci (*BAT25* and *D5S346*) and was classified MSI-H. For the other patient with MSI at the *BAT25* locus, there was only one tumor to evaluate. We examined a second tumor from the third patient displaying MSI at *D17S250*, and did not find MSI in the second focus. This latter tumor along with the other remaining tumors which did not display MSI at any of the five loci were classified as MSI-L/MSS.

The overall conclusion from this study is that PC does not display a high level of microsatellite instability when the NCI panel and criteria described above are used. We observed MSI in at least one tumor for 3/16 cases (19%), with one of these cases having two tumors with MSI (one being MSI-H and one being MSI-L). However, the number of microsatellite markers displaying instability was low relative to tumors from HNPCC where the number of positive markers was found, on the average, to be around 80% in our laboratory. The finding that MSI was seen in two tumors from the same patient suggests the possibility of an underlying genetic defect.

Task 4 **Final Analysis and Preparation of Reports.** (Months 25-30)

A. Final quality control checks for epidemiologic, clinical, and laboratory data have been conducted.

B. Biomarker data are being integrated with epidemiologic and clinical data.

C. The final report has been prepared and is being submitted.

D. We have published three manuscripts, one has been submitted and two others are currently being prepared for submission. We have had five abstracts accepted for presentation at research meeting as listed below.

KEY RESEARCH ACCOMPLISHMENTS

- Data generated from this proposal were included as preliminary data in recently funded prostate cancer SPORE grant.
- 140 patients newly diagnosed with metastatic prostate cancer have been accrued for this study
- Epidemiologic data and biological samples (blood and prostate tissue) have been collected for study participants
- Gene expression assays have been established and utilized.
- 167 (70 cases and 97 controls) samples have been processed for gene expression assays.
- Genotyping has been completed for androgen receptor, vitamin D receptor and 5-α-reductase for study participants.
- MSI analyses have been completed in a sub-sample of tumor and normal tissue.

REPORTABLE OUTCOME

- 3 manuscripts have been published.
- 1 manuscript has been submitted (Appendix A).
- 2 manuscripts are being prepared for publication
- Five abstracts have been presented at different scientific meetings.

CONCLUSIONS

Our findings suggest that decreased mismatch repair gene expression may be associated with increased risk of prostate cancer. These results suggest that DNA damage-repair pathways may be involved in prostate carcinogenesis. Results from the analyses of the genetic polymorphisms suggest that some of these may also play a role in prostate cancer prognosis. Further and larger studies are needed to confirm these findings and to further explore the molecular basis of the underlying mechanisms of prostate cancer etiology and progression.

Abstracts & Manuscripts published

Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC, Hurting SD. *Phytoestrogen Intake and Prostate Cancer: A case-control study using a new database.* Nutrition & Cancer. *33(1): 20-25, 1999.*

Spitz MR, Strom SS, Yamamura Y, Troncoso P, Babaian RJ, Scardino PT, Wheeler T, Amos CI, von Eschenbach A, Kagan J. *Epidemiologic Determinants of Clinically Relevant Prostate Cancer.* Int J Cancer 89, 259-264, 2000.

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Strom SS, Zhang Q, Gu Y, Spitz MR, Scardino PT, Logothetis CJ, Pettway CA, Wei Q. *Lack of Association between a Vitamin D Receptor Polymorphism and Prostate Cancer Risk.* (Submitted).

Strom SS, Spitz MR, Guan Y, Yamamura Y, Babaian RJ, Scardino P, Wei Q. *Reduced Expression of DNA-Repair Related Genes in Prostate Cancer.* AACR, 1998.

Strom SS, Duphorne CM, Yamamura Y, Hursting SD, Spitz MR. *Sources of Phytoestrogens in the American Diet.* ASPO, 2000.

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Yamamura Y, Frazier M, Spitz MR, Babaian RJ, Logothetis CJ, Troncoso P, Strom SS. *Role of Cyclin D1Polymorphisms in Prostate Cancer: Racial Differences in Genotypic Distribution.* AACR, 2002.

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APPENDIX A

Strom SS, Zhang Q, Gu Y, Spitz MR, Scardino PT, Logothetis CJ, Pettaway CA, Wei Q. *Lack of Association Between a Vitamin D Receptor Polymorphism and Prostate Cancer Risk.* (submitted manuscript)

Lack of Association between a Vitamin D Receptor Polymorphism and Prostate Cancer Risk [1]

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Introduction

Vitamin D has potent antitumor properties. In vitro and in vivo laboratory studies of prostate cancer (PC) have demonstrated that vitamin D metabolites [e.g., $1,25(OH)_2D_3$] and its analogues have strong prodifferentiative and growth-inhibitory effects (1). The effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR) which is expressed by both normal and malignant prostate cells (1). The VDR gene has been found to have several polymorphic regions in the 3'untranslated region (3'UTR) including the polymorphism we investigated, TaqI (2, 3). Previous studies have reported positive (2), protective (3) and null (4-7) associations between this polymorphism and PC risk. Based on these inconclusive data, we evaluated the genotype frequency in a large case-control study to test the hypothesis that men with the tt genotype are less likely to be diagnosed with PC. To the best of our knowledge, this is the largest case-control study ever done to evaluate the association between vitamin D receptor polymorphism and prostate cancer risk.

Materials and Methods

As previously described (8), cases are 407 white men diagnosed with histologically confirmed adenocarcinoma of the prostate referred to the University of Texas M. D. Anderson Cancer Center (MDACC) and Baylor College of Medicine. No case had a prior history of invasive cancer.

Age and ethnicity matched controls (n = 403) were selected from men who attended the MDACC prostate cancer screening clinic. Men with elevated prostate-specific antigen

(PSA) levels (PSA $\geq$ 4 ng/ml), an abnormal digital rectal examination, or previous history of cancer were excluded from the study. Demographic and risk factor information was obtained through personal interviews. Patient clinical information was abstracted from medical record review. DNA was extracted from blood samples collected from each participant.

VDR TaqI genotype was determined by a PCR-based method described by Riggs et al (9). A 740 bp fragment located within intron 8 and exon 9 was generated using PCR primers (sense, 5'- CAG AGC ATG GAC AGG GAG CAA and antisense, 5'-GCA ACT CCT CAT GGC TGA GGT CTC). The PCR fragment was subjected to TaqI digestion and then separated on 3% Nusieve 3:1 agarose gel (FMC Bioproducts Rockland, ME). Based on varied fragment lengths, the genotype was assigned as following: TT, fragments 495-bp and 245-bp; Tt, fragments 495-bp, 290-bp, 245-bp and 205-bp; and tt, fragments 290-bp, 245-bp and 205-bp.

Unconditional multiple logistic regression models were used to evaluate the association between VDR genotype and PC risk. The model was adjusted for family history of prostate cancer among first degree relatives (i.e., father, brother and son).

Results

Allele frequencies and genotypes of TaqI were compatible with Hardy-Weinberg equilibrium in both controls (Observed vs. expected: $\chi^2 = 1.20$, $p = 0.55$) and cases ($\chi^2 = 0.77$, $p = 0.68$). We did not find any significant association between TaqI T allele and PC

risk after adjusting for age and family history of prostate cancer in our models. We compared participants with TT and Tt genotype to those with tt genotype controlling for age and family history (Table 1). The odds ratio (OR) for (TT + Tt) vs. tt was 1.25 [95% confidence interval (CI): 0.87 – 1.81]. Although the OR was slightly higher in the younger age group (≤ 55 years) as compared to the older group (> 55 years) (OR = 1.59, p = 0.31 and OR = 1.21, p = 0.35, respectively), the difference was not significant. We did find suggestive evidence associating the presence of the T allele with advanced as compared to locally confined disease (OR = 1.36, p = 0.28 and OR = 1.22, p = 0.34, respectively), however, again, these results were not statistically significant.

Discussion

Since vitamin D has been reported to be involved in the regulation of cell proliferation and differentiation in vitro (10), polymorphisms in the vitamin D receptor gene were prime candidates for explaining genetic differences in the response to vitamin D. In this large case-control study, we found no significant association of TaqI with prostate cancer risk overall, nor in relation to age, or positive family history of PC. Our results are consistent with previous null findings (4-7). The non-significant association with advanced disease suggests that disease progression may be influenced by VDR rather than the cancer initiation. Our sample size was sufficient to detect a minimum relative risk of 2.0 with 80% power, assuming a significance level of 0.05, based on the genotype frequency of the controls. In conclusion, our data indicate that VDR polymorphism TaqI is not a strong independent predictor for prostate cancer risk.

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Table 1 Association of VDR genotype with the risk of prostate cancer

Genotype	Cases (n = 407) No.	(%)	Controls (n = 403) No.	(%)	OR	95% CI
All subjects						
tt	69	(17.0)	79	(19.6)	1.0	
Tt	182	(44.7)	181	(44.9)	1.19 [a]	0.81 – 1.76
TT	156	(38.3)	143	(35.5)	1.33 [a]	0.89 – 1.99
(TT + Tt) vs. tt					1.25 [a]	0.87 – 1.81
Age ≤ 55 years						
tt	10	(13.3)	15	(16.9)	1.0	
Tt	35	(46.7)	41	(46.0)	1.55 [b]	0.60 – 4.03
TT	30	(40.0)	33	(37.1)	1.62 [b]	0.62 – 4.27
(TT + Tt) vs. tt					1.59 [b]	0.65 – 3.89
Age > 55 years						
tt	59	(17.8)	64	(20.4)	1.0	
Tt	147	(44.3)	140	(44.6)	1.14 [b]	0.74 – 1.75
TT	126	(37.9)	110	(35.0)	1.30 [b]	0.83 – 2.02
(TT + Tt) vs. tt					1.21 [b]	0.81 – 1.80

[a] Adjusted for age and family history of PC.
[b] Adjusted for family history.

APPENDIX B

Yamamura Y, Thompson PH, Spitz MR, Babaian RJ, Logothetis CJ, Strom SS. *Role of 5-alpha-Reductase Polymorphisms in Prostate Cancer Prognosis*. AACR 2001

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ROLE OF 5-α-REDUCTASE POLYMORPHISMS IN PROSTATE CANCER PROGNOSIS

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Cell growth in the human prostate gland is regulated by androgens. The protein product of the SRD5A2, 5-α -reductase type II gene, is responsible for the conversion of testosterone to the more bioactive form dihydrotestosterone. Several studies have indicated that this gene may play a role in the development of prostate cancer (PC) with cases being less likely to have the Leu/Leu genotype than ethnicity- and age-matched healthy men. We evaluated the association of a SRD5A2 genetic polymorphism with known clinical prognostic indicators among 233 Caucasian men with all stages of PC enrolled in an ongoing PC study conducted at the University of Texas M.D. Anderson Cancer Center. Specifically, DNA from peripheral lymphocytes was isolated and genotyped for the single nucleotide polymorphism in the SRD5A2 gene that results in the substitution of valine for leucine at codon 89 (V89L). Overall, 7.3% of our patients had the Leu/Leu genotype. Although men with the Leu/Leu genotype tended to be younger than those with Val/Val or Val/Leu genotypes (62.4 vs. 61.4 years, respectively), this difference was not statistically significant (p=0.5). There was no association between genotype and combined Gleason score. Similarly, we found no relationship between genotype and having metastatic disease. Our results do not support a role for the V89L mutation in the SRD5A2 gene in PC prognosis. We are in the process of genotyping these same cases for the mis-sense substitution which results in an alanine residue at codon 49 being replaced by threonine (A49T); we believe that this polymorphism may play a more significant role in PC progression.
(Supported by NIH grant CA/ES68578 & CA84964, and DOD grant DAMD 17-98-1-84)

APPENDIX C

Strom SS, Spitz MR, Yamamura Y, Babaian RJ, Scardino PT, Wei Q. *Reduced Expression of hMHS2 and hMLH1 and Risk of Prostate Cancer: A Case-Control Study.* The Prostate 47: 269-275, 2001.

The Prostate 47:269–275 (2001)

Reduced Expression of *hMSH2* and *hMLHI* and Risk of Prostate Cancer: A Case-Control Study

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BACKGROUND. Although prostate cancer is the most common incident cancer in men, not much is known about its etiology. We tested the hypothesis that expression levels of *hMSH2* and *hMLH1* in unaffected (normal) tissue play a role in the etiology of prostate cancer.
METHODS. Total RNA was extracted from peripheral blood lymphocytes of subjects ascertained by a case-control study (70 patients and 97 age- and ethnicity-matched controls). A multiplex reverse transcription-polymerase chain reaction assay was used to simultaneously evaluate the relative expression of *hMSH2* and *hMLH1*, using β-actin as the internal control.
RESULTS. The relative gene expression levels of *hMSH2* and *hMLH1* were significantly lower in cases than in controls ($P < 0.05$ for both genes). When compared with the highest tertile of the controls, low expression levels (the middle and lowest tertiles) of *hMLH1* were associated with significantly increased risk of prostate cancer in a dose–response relationship (ORs = 2.68, and 4.31; 95% confidence interval = 1.00–7.23 and 1.64–11.30, respectively) after adjustment for age, ethnicity, smoking status, and family history of prostate cancer.
CONCLUSIONS. These results suggest that reduced expression of *hMLH1* in peripheral lymphocytes may be a risk factor for prostate cancer. However, it cannot be ruled out that the reduced expression we observed may be caused by the disease status. Our findings and the factors that may affect the expression of *hMLH1* need further confirmation in larger prospective studies. *Prostate 47:269–275, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: **biomarker; gene expression; mismatch repair; molecular epidemiology; polymerase chain reaction**

INTRODUCTION

Prostate cancer is the most common incident cancer in men; yet little is known about its etiology. Although mutations and polymorphisms in androgen receptor genes suggest that male hormones may play a role in the etiology of prostate cancer [1], genetic determinants of prostate cancer remain largely unknown. Abnormalities in several genes, including tumor suppressor genes such as *p53* [2], *MXI1* [3] and *PTEN/MMAC1* [4], and cell-cycle related genes such as *RB* [5] and *p16* [6], have been identified in prostate tumors, but their mutation frequencies are relatively rare. As normal cell-cycle arrest is necessary for DNA repair [7],

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; OR, odds ratio; CI, confidence interval; MIN, microsatellite instability.

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somatic mutations and genetic alterations in prostate cancer tumors in genes controlling cell-cycle progression and cell growth suggest that abnormalities in DNA repair may be involved in prostate cancer etiology.

It has been suggested that a mutator phenotype may be involved in the development of cancer [8]. This phenotype is characterized by microsatellite instability (MSI) as a result of deficiencies in DNA mismatch repair [9,10]. In prostate cancer, MSI, known as dinucleotide tandem repeat sequences, has been found in up to 45% of tumors [11–13]. Studies suggest that MSI may be an early event in prostate carcinogenesis, but not a marker for progression or prognosis [14,15]. Although MSI is frequently found in prostate cancer, mutations in two major mismatch repair genes *hMSH2* and *hMLH1* are relatively rare, and studies of these genes have been limited to a few established prostate cell lines [16,17]. It has been recently postulated that the methylation status of *hMLH1*, particularly in its promoter region, regulates gene expression and is linked to MSI [18–21]. This finding suggests that aberrant gene expression may be epigenetic and can be caused by factors other than mutations in the genes.

There are no published data on an association between MSI and methylation status of *hMSH2* and *hMLH1* or expression level of these two genes in prostate cancer. Epidemiologically, expression levels of *hMSH2* and *hMLH1* in unaffected (normal) tissues of prostate cancer patients and their role in the etiology of prostate cancer are of interest. To evaluate the expression levels of *hMSH2* and *hMLH1* simultaneously, we modified our previously published multiplex reverse transcription-polymerase chain reaction assay [22,23] to measure the relative expression of these two genes in peripheral blood lymphocytes as a surrogate tissue. We then conducted a pilot case-control study to test the hypothesis that low expression levels of *hMSH2* and *hMLH1* are associated with increased risk of prostate cancer. In this report, we describe a significant association between low expression of *hMLH1* and increased risk of prostate cancer in a study of 70 prostate cancer patients treated with radical prostatectomy and 97 healthy controls.

MATERIALS AND METHODS

Study Population

The cases were patients registered at The University of Texas M.D. Anderson Cancer Center or Baylor College of Medicine with histologically confirmed adenocarcinoma of the prostate. Men with metastatic prostate cancer or a previous history of invasive cancer were excluded from the study. The subjects included in this analysis had not had any treatment for prostate cancer other than prostatectomy. These men were participants enrolled between 1997 and 1998 in an ongoing molecular epidemiologic case–case study. The controls were identified from two sources. The first group of participants (75%) was selected from men attending the M.D. Anderson Cancer Center prostate cancer-screening program. Men who had prostate-specific antigen levels $\geq$4 mg/ml, an abnormal rectal digital examination, or previous history of cancer were excluded. The second group of controls (25%) was selected from among male members of a large multi-specialty managed care organization. Only subjects without a history of cancer or urological conditions were included as controls. The cases and controls were matched on age ($\pm$5 years) and ethnicity. After written informed consent was obtained, each participant donated 10 ml of blood collected in heparinized tubes and completed either a personal or phone interview that assessed demographic and risk-factor information and family history of prostate cancer.

Multiplex RT-PCR

Because we have found that it is difficult to extract sufficient RNA from blood samples drawn more than 24 hr before processing, we extracted total RNA from all the samples that had been processed with the Tri-Reagent, a RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH), within 8 hr of procurement in this study. On each sample, we performed a multiplex RT-PCR assay using the β-actin gene as an internal control [22,23] to evaluate simultaneously the expression of *hMSH2* and *hMLH1*. The inclusion of the internal control β-actin allowed us to evaluate contamination of genomic DNA and to normalize variation in the amount of RNA used for cDNA synthesis as well as the amount of PCR product loaded on gels. To amplify these two selected genes, we used our previously published multiplex RT-PCR protocol, in which the strategy for choosing the primers has been described [22,23]. The sequences of the primers were 5′-ACACTGTGCCCATCTACGAGG-3′ (sense) and 5′-AGGGGCCGGACTCGTCATACT-3′ (antisense) for β-actin (GenBank accession no. M10277; starting positions 2147 and 2954, respectively); 5′-GTCGGCTTCGTGCGCTTCTTT-3′ (sense) and 5′-TCTCTGGCCATCAACTGCGGA-3′ (antisense) for *hMSH2* (U03911; starting positions 52 and 460, respectively); and 5′-GTGCTGGCAATCAAGGGACCC-3′ (sense) and 5′-CACGGTTGAGGCATTGGGTAG-3′ (antisense) for *hMLH1* (U07418; starting positions 466 and 660, respectively).

Briefly, cDNA was synthesized by RT with 0.5 μg of random primers (Promega Biotech, Piscataway, NJ), 200 U of Moloney murine leukemia virus reverse transcriptase (United States Biochemical Co., Cleveland, OH), 1 μg of total cellular RNA, 4 μl of 5 × RT buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 50 mM dithiotheitol; and 15 mM $MgCl_2$; Life Technologies, Gaithersburg, MD), 0.25 mM each dNTP, 20 units of RNasin (Promega Biotech), and 6.5 μl of diethyl pyrocarbonate-treated water. The 20-μl reaction mixtures were incubated at room temperature for 10 min and at 42°C for 45 min, heated to 90°C for 10 min, and then quickly chilled on ice.

The PCR primer mixture was optimized by experimenting with different combinations of concentrations of each pair of primers to produce a clearly visible band for each of the genes on an agarose gel. The optimal 50-μl PCR mixture contained 3-5 μL of RT reaction mixture, 1 × PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0; 1% Triton X-100; and 2.5 mM $MgCl_2$), 0.04 mM each dNTP, 2 U of *Taq* polymerase (Promega Biotech), 25 pM β-actin primers, 125 pM *hMSH2* primers and 20 pM *hMLH1* primers. The mixtures were amplified with a Perkin-Elmer GeneAmp PCR System 2400 (Foster City, CA) by an initial denaturation step of 95°C for 5 min; 29 cycles of denaturation at 95°C for 30 sec, primer annealing at 59°C for 30 sec, and extension at 72°C for 45 sec; and a final elongation step at 72°C for 10 min. This optimal protocol allows the amplification of all genes simultaneously in 29 cycles and gave very consistent results in repeated assays [21]. The assays were performed in batches of 6–8 samples with equal numbers of cases and controls. The RT-PCR products were separated by 1.5% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, visualized with ultraviolet light, and captured as an electronic photo [Fig. 1]. The bands on the photos were then scanned as digitized images, and the areas under the peaks were calculated in arbitrary units by densitometric analysis with a computerized Digital Imaging System (Model IS-1000; Alpha Innotech Co., San Leandro, CA). The internal standard (β-actin) in each reaction was used as the baseline gene expression of that sample. The relative expression value for each of the target genes amplified in that reaction was calculated relative to the β-actin value (100%). These values were then compared across the samples tested. Reduced expression was verified by repeating the multiplex RT-PCR assay.

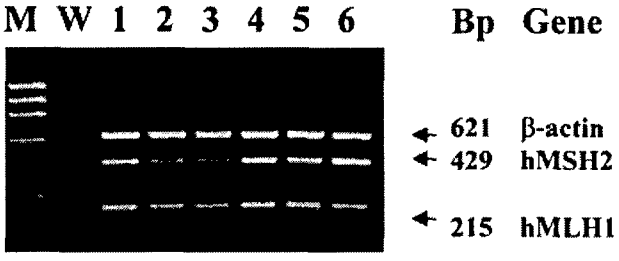

Fig. 1. Detection of expression of *hMSH2* and *hMLH1* in a batch of blood samples. This is a gel photo processed by a computerized imaging system. MW, molecular weight marker (øX174 RF DNA/*Hae*III); lanes 1–3, three cases; lanes 4–6, three controls.

Tumor-Related Measurements

A Combined Gleason Score describing the histological differentiation pattern of a tumor was recorded for each patient. Plasma samples were used to measure testosterone levels by enzyme-linked immunosorbent assays and dihydrotestosterone by radioimmunoassay in a subset of case participants. Both hormones were measured using commercially available kits from Diagnostics Systems Laboratories Inc. (Webster, TX).

Statistical Analysis

Chi-square tests were used to assess differences in the distribution of demographic variables. The difference in the expression level of each gene (as a continuous variable) between cases and controls was evaluated by using Student *t*-test. Pearson correlation coefficients were calculated to evaluate correlation among demographic factors, smoking status, alcohol use, family history of prostate cancer, testosterone levels, and gene expression values. Crude odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for demographic variables, smoking status, family history of prostate cancer in first degree relatives (father, brother or son), and gene expression levels. Men who smoked more than 100 cigarettes in their lifetimes were considered "ever smokers." Gene expression values were then categorized into tertiles based on the expression level of the controls, and the ORs were estimated as the risks of those in the lowest tertile compared with those in the highest tertile of gene expression. The ORs were also adjusted for important covariates such as age, smoking status, and family history of prostate cancer in the multivariate logistic regression analysis. To perform the linear trend test, the tertile dummy variables were recoded as one continuous variable (1 to 3 for the lowest to highest tertile) and fitted into a logistic regression model with and without adjustment for the covariates. All statistical tests were two-sided and were performed with Statistical Analysis System software (Version 6; SAS Institute Inc., Cary, NC).

RESULTS

There were 70 cases and 97 controls included in this study (Table I). These men were primarily non-Hispanic whites (91%). The frequency matching resulted in no statistically significant differences in the distributions of age and ethnicity between cases and controls ($P = 0.769$ and $P = 0.332$, respectively). The mean age of the cases (mean±SD, 64.4±7.5 years) was higher than that of the controls (63.1±7.0 years), but this difference was not statistically significant ($P = 0.252$) (Table II). A higher percentage of cases (76%) than controls (57%) reported being ever smokers ($P = 0.017$), whereas the prevalence of current drinking was similar in cases (57%) and controls (53%) ($P = 0.648$). More cases (21%) than controls (6%) reported having a first-degree relative (father, brother, or son) with prostate cancer ($P < 0.001$) (Table I). Therefore, the important covariates including age, smoking status, and family history of cancer were further adjusted for in multivariate logistic regression analysis.

For both cases and controls, there were considerable variations in the expression levels of *hMSH2* and *hMLH1*, but the mean expression level was statistically significantly lower in the cases than in the controls for both *hMSH2* and *hMLH1* ($P = 0.016$ and $P = 0.003$, respectively) (Table II). The expression levels of these two genes were not significantly correlated with age or smoking status, in either the cases or the controls (data not shown), suggesting that these factors did not have an effect on the expression levels. Furthermore, there

TABLE I. Distribution of Selected Variables in Prostate Cancer Cases and Healthy Controls

	Cases		Controls		
Variable	Number	(%)	Number	(%)	*P* value[a]
Total	70	(100)	97	(100)	
Age (in yr)					
≤65	42	(60)	56	(58)	
>65	28	(40)	41	(42)	0.769
Ethnicity					
Non-Hispanic white	64	(91)	84	(87)	
Others	6	(9)	13	(13)	0.332
Smoking[b]					
Ever	53	(76)	48	(57)	
Never	17	(24)	35	(43)	0.017
Alcohol use[b]					
Current user	40	(57)	46	(53)	
Former or never user	30	(43)	40	(47)	0.648
Family history of prostate cancer					
Yes	15	(21)	6	(6)	
No	55	(79)	91	(94)	0.003

[a]Chi-square test for distribution.
[b]Numbers do not add up to total number of the controls because of missing information.

TABLE II. Differences in Age and Gene Expression of *hMSH2* and *hMLH1* between Prostate Cancer Cases and Healthy Controls

	Mean (%)±SD			
	Cases (n = 70)	Controls (n = 97)	% Difference[a]	*P* value[b]
Age (in yr)	64.4 ± 7.5	63.1 ± 7.0		0.252
Gene				
hMSH2	35.6 ± 22.2	44.0 ± 22.0	−19.1	0.016
hMLH1	41.0 ± 13.8	47.8 ± 14.5	−14.2	0.003

[a]% Difference = $[(\text{Expression}_{case} - \text{Expression}_{control})/\text{Expression}_{control}] \times 100\%$.
[b]Two-sided *t* test.

TABLE III. Logistic Regression Analysis for Gene Expression Levels of *hMSH2* and *hMLH1* in Prostate Cancer and Healthy Controls

Gene expression level[a]	Number		Crude OR (95% CI)	Adjusted OR[b] (95% CI)
	Cases	Controls		
hMSH2				
HT	17	32	1.00	1.00
MT	15	33	0.86 (0.37–2.00)	0.65 (0.26–1.62)
LT	38	32	2.24 (1.05–4.75)	1.81 (0.80–4.12)
Trend test[b]			$P = 0.023$	$P = 0.101$
hMLH1				
HT	8	32	1.00	1.00
MT	25	33	3.03 (1.19–7.70)	2.68 (1.00–7.23)
LT	37	32	4.62 (1.87–11.46)	4.31 (1.64–11.30)
Trend test[b]			$P = 0.001$	$P = 0.004$

[a]HT, the highest tertile; MT, the middle tertile; LT, lowest tertile; based on controls levels.
[b]Adjusted for age, smoking status, and family history of prostate cancer in a logistic regression model.

was no correlation between expression levels of these two genes ($r = 0.02$, $P = 0.76$).

We explored the correlation between serum testosterone and dihydrotestosterone levels and gene expression among the cases. We found a borderline correlation between the expression level of *hMSH2* and testosterone ($r = -0.26$, $P = 0.06$), but no correlation between the expression level of *hMLH1* and testosterone ($r = -0.07$, $P = 0.62$). We also explored the relationship between the combined Gleason Score and the expression levels. Cases who had a score 7 and above had similar expression levels of the two genes compared with cases who had a score less than 7 (data not shown). These data indicate that the gene expression was not influenced by hormone level or tumor status.

Crude and adjusted ORs were derived from logistic regression analysis and are shown in Table III. The adjusted results did not differ substantially from the crude ORs. Only reduced expression of *hMHl1* remained a statistically significant risk factor for prostate cancer (OR = 4.31, $P = 0.004$) when the lowest and highest tertiles were compared with adjustment for age, smoking status, and family history of prostate cancer.

DISCUSSION

In this case–control study, we evaluated the association between the relative gene expression levels of *hMSH2* and *hMLH1* and prostate cancer risk by using a multiplex RT-PCR assay. We found an association between reduced expression of *hMLH1* and prostate cancer risk that was independent of age, smoking status or family history of prostate cancer. Although the reduction in expression of these genes varied from individual to individual, there was reduced expression of both genes in cases compared with healthy controls. Because the patients were newly diagnosed with prostate cancer and gene expression was measured in peripheral lymphocytes that were unlikely affected by disease status, the findings support our hypothesis that reduced mismatch gene expression may be associated with risk of prostate cancer.

To the best of our knowledge, data on mutations of *hMLH1* and *hMSH2* in prostate cancer tumor tissue have not been previously reported, although some studies of prostate cell lines have exhibited MSI and deficient mismatch repair [16,17]. While there are no data that demonstrate a link between MSI and mutations in *hMLH1* and *hMSH2* or between MSI and the expression levels of these two genes in prostate cancer, there are several lines of evidence that suggest that aberrant expression of these genes may be involved in human carcinogenesis.

It is possible that changes in gene expression can be influenced by both genetic and epigenetic factors and may play a role in carcinogenesis [24], and changes in the expression of *hMLH1* and *hMSH2* may be associated with alterations of mismatch repair function [18,25]. Defective mismatch repair is believed to be responsible for MSI [9,10]. Several studies have analyzed MSI in prostate cancer and have reported frequencies up to 45% [11–13]. Recently, the overall rarity of mutations of *hMLH1* and its relatively frequent aberrant expression seen in tumors are shown to be associated with hypermethylation status

of this gene [18–20]. In contrast, the cancers displaying hMLH1-protein expression were not methylated in the *hMLH1*-promoter region [20]. These data suggest that there is a significant association between the *hMLH1* expression level and genetically or epigenetically controlled level of hypermethylation of its promoter in tumors with MSI. Our data in this report are consistent with previous findings of reduced expression of *hMLH1* in gliomas with MSI [26,27] and in lymphocytes of head and neck cancer [28] and colon cancer patients [29]. Taken together, available data support our finding that low expression of *hMLH1* is a risk factor of cancer and may play a role in the etiology of prostate cancer.

The use of unstimulated peripheral blood lymphocytes as the surrogate tissue in this study has advantages and disadvantages, particularly in measuring gene expression. Although lymphocytes may have been exposed to the same endogenous and exogenous chemical carcinogens as the target organ (the prostate), as blood serves as the carrier, the extent of exposure to endogenous DNA-damaging agents may be different due to local metabolic processes. Although unstimulated lymphocytes may provide information about the genetic background of gene expression, it is well known that there is almost no nucleotide excision repair activity in unstimulated peripheral blood lymphocytes [30]. We have also noted that expression of the *p27*, *RAD51*, and *PCNA* genes was not detectable in unstimulated lymphocytes under our RT-PCR conditions (unpublished data). Although we have demonstrated that the expression levels of several nucleotide repair genes in both stimulated and unstimulated lymphocytes were similar [31], the expression levels of *hMLH1* or *hMSH2* in stimulated and unstimulated lymphocytes should be compared in future studies.

In conclusion, our findings show that reduced expression of *hMLH1* in lymphocytes was associated with increased risk of prostate cancer, suggesting that DNA damage-repair pathways may be involved in prostate carcinogenesis. However, it cannot be ruled out that the reduced expression we observed may be caused by the disease status. Our findings and the factors that may affect the expression of *hMLH1* need further confirmation in larger prospective studies with prediagnostic specimen and in prostate tissue. There is also a need to further explore the molecular basis of the underlying mechanism of such low expression. Studies of associations between MSI and expression level or hypermethylation of mismatch repair genes in prostate tumors and between hypermethylation and expression level of mismatch repair genes in normal tissues are critical for illustrating their involvement in the etiology of prostate cancer.

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